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Differences Between Enzymatic and Diazo Methods for Measuring Direct Bilirubin

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Summary: An orthodox diazo method is popularly used for measuring bilirubin. On the other hand, an enzymatic method which employs bilirubin oxidase, has also been in use for considerable time. We have often found disparities between direct bilirubin values obtained with the enzymatic and the diazo methods. To determine the cause of these disparities, bilirubin subfractions were analysed and classified into two types by HPLC. Samples showing great differences contained conjugated, unconjugated and δ bilirubins (type I), while samples showing only small differences contained almost exclusively unconjugated bilirubin and δ bilirubin (type II). Conjugated bilirubin is therefore largely responsible for the differences observed between the two methods. Particularly marked differences were found for bile (in which all the bilirubin is conjugated) and for synthetic conjugated bilirubin. Bilirubin oxidase decreases the absorbance at 450 nm when it catalyses oxidation of bilirubin, but after the oxidation of synthetic conjugated bilirubin at pH 3.7 another peak appeared at 450 nm, as shown by HPLC and spectrophotometry, but not when the reaction was performed at pH 7.2, namely under conditions permitting complete oxidation. Incomplete oxidation products of conjugated bilirubin are responsible for the disparity. Care is therefore needed in the clinical interpretation of direct bilirubin values measured by the enzymatic method.

Introduction

Orthodox diazo methods, such as the *Malloy-Evelyn* method (1), are popular for the measurement of bilirubin. An enzymatic method (2, 3) is also used for measuring total and direct bilirubin, based on the decrease in absorbance at 450 nm when bilirubin is oxidized to biliverdin in the presence of bilirubin oxidase (4, 5).[1]) But this is not a complete replacement for the diazo method, because large differences in direct bilirubin values are often found between the two methods, while the values for total bilirubin are largely in agreement.

The aim of this study is to clarify the cause of this difference. First hyperdirectbilirubinaemia was divided into two types. Type I showed large differences, while type II showed only small differences. The bilirubin subfractions of these two types were investigated by HPLC.

In addition, ditaurobilirubin (6), a synthetic conjugated bilirubin, was analysed by the bilirubin oxidase reaction, by HPLC and by spectrophotometry.

In the present study HPLC proved to be a potent tool for analysing bilirubin subfractions and for identifying the sources of absorbance changes in bilirubin oxidation.

Materials and Methods

An HPLC, LC-6A (Shimadzu Corporation, Kyoto, Japan) was used with a Micronex RP-30 column (Sekisui Chemical Co., Osaka, Japan) for bilirubin subfractionation. The HITACHI 736-60 (HITACHI, Ltd., Tokyo, Japan) was used for spectrophotometric measurements.

Icteric sera (total bilirubin ≧ 34 μmol/l, direct bilirubin ≧ 11 μmol/l) were collected at random from the laboratory of Niigata University Hospital. The sera were neither haemolysed nor chylous.

Bile was obtained from an otherwise normal gall-stone patient during his operation. It was then filtered through a MILLEX-HV 0.45 μm Filter Unit (Millipore Corporation, Bedford, MA USA).

[1]) Enzyme: Bilirubin oxidase (EC 1.3.3.5)

Bilirubin-C (ditaurobilirubin) of "Interference Check-A" (INTERNATIONAL REAGENTS CORPORATION, Kobe, Japan) served as conjugated bilirubin.

HPLC subfractionation

HPLC subfractionation was done as described by *Adachi* et al. (7). Briefly, a sample was mixed with an equal amount of 0.1 mol/l acetic acid reagent, then passed through a MILLEX-HV 0.45 µm filter to eliminate any fibrin precipitates. A 20-µl aliquot of this filtrate was injected into the HPLC. Finally, the absorbance was measured at 450 nm. Assay variation was 7.7% (n = 5) within-day, 3.5% (n = 10) between-run.

Diazo method

An alkaline azobilirubin method kit, Nescauto BIL-V3 (Nippon Shoji Kaisha, Ltd., Osaka, Japan) (8), was used for the diazo method. The diazo reagent of this kit contains sulphanilic acid and sodium nitrite dissolved in hydrochloric acid solution, as in the *Malloy-Evelyn* reagent, but the accelerator consists of urea, sodium acetate and glycine. The absorbance gain was detected at 456 nm on an autoanalyser HITACHI 736-60. The intra-assay and the inter-assay precision of the total bilirubin assay were 2.9% and 3.7% respectively, whereas those of the direct bilirubin assay were 2.3% and 2.4%, respectively, for the analysis of (n = 4) serum samples.

Enzymatic method

An enzymatic method kit, Nescauto BIL-VE (Nippon Shoji Kaisha, Ltd., Osaka, Japan) (9) was used, and decreases at 450 nm were measured on a HITACHI 736–60. The intra-assay and the inter-assay precision for total bilirubin were 2.7% and 2.0%, respectively, whereas those for direct bilirubin were 3.7% and 4.5%, respectively, for the analysis of (n = 4) serum samples.

Results

Bilirubin subfractionation in serum

Figure 1a shows four bilirubin subfractions detected on HPLC: α (unconjugated bilirubin), β (monoconjugated bilirubin), γ (biconjugated bilirubin) and δ (δ bilirubin (10–12), which is a covalently albumin-bound bilirubin). Their retention times (mean ± SD) were 34.7 ± 0.1 min, 29.3 ± 0.1 min & 28.6 ± 0.1 min (biphasic), 24.9 ± 0.1 min, and 20.2 ± 0.3 min, respectively.

Comparison of bilirubin values

Figure 2b shows the direct bilirubin values of eight serum samples that show large differences between the two methods. The average difference ($|\text{direct bilirubin}_{diazo} - \text{direct bilirubin}_{enzymatic}|$)/direct bilirubin$_{enzymatic}$ was 53%. The average difference for total bilirubin ($|\text{total bilirubin}_{diazo} - \text{total bilirubin}_{enzymatic}|$)/total bilirubin$_{enzymatic}$ was 7% as shown in figure 2a. Their chromatograms always show all the four main peaks (fig. 1a) (type I). Conversely, figure 3 shows three serum samples that show only small differences between the two methods (type II); the average differences for direct and total bilirubin were 14% and 3%, respectively.

Bilirubin subfractionation in bile

Figure 1c shows a chromatogram of bile. Its chief constituents were β (retention time: 28.4 min & 29.6 min)

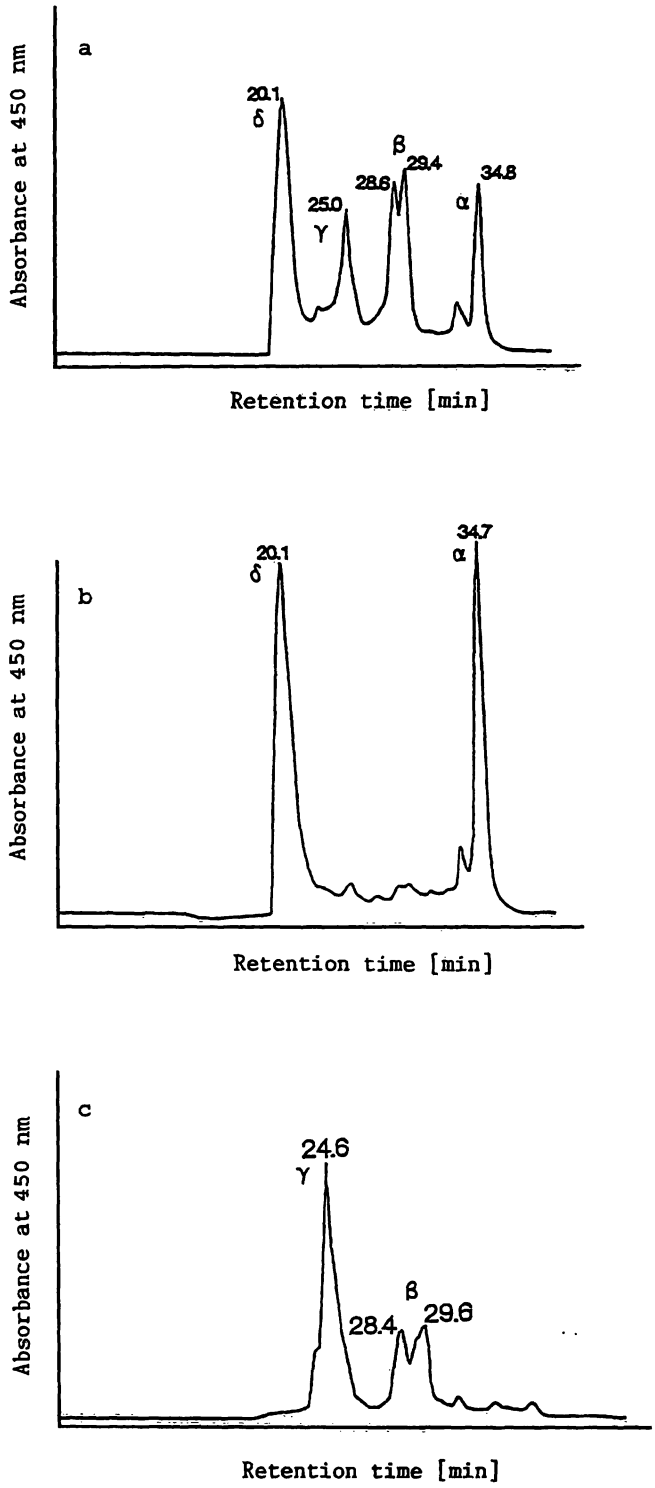

Fig. 1 Chromatogram of icteric sera and bile.
(a) Sera showing large methodical differences.
(b) Sera showing small differences.
(c) Bile.
The number and Greek letter at each peak indicate the retention time (min) and the subfraction name, respectively.

and γ (retention time: 24.6 min). The difference between the two methods was substantial (data not shown).

Absorbance changes of ditaurobilirubin before and after each bilirubin assay

To investigate the disparity in direct bilirubin values, ditaurobilirubin was used as a conjugated bilirubin. The absorbance due to ditaurobilirubin was detected in different concentrations before and after the reaction (measured on the autoanalyzer HITACHI 736-60 for the diazo and enzymatic reactions at 546 nm and at 450 nm, respectively). Figure 4 indicates that the baseline of the enzymatic reaction rose according to the concentration of ditaurobilirubin, while that of the diazo method was almost flat.

Changes in chromatographic behaviour due to the bilirubin oxidase reaction

The chromatograms of ditaurobilirubin before and after the addition of bilirubin oxidase at pH 3.7 and pH 7.2 were compared. At pH 3.7 (for direct bilirubin measurement), the β and γ peaks completely disappeared after addition of bilirubin oxidase (fig. 5), but other material absorbing at 450 nm appeared during the retention time 2.9 to 3.5

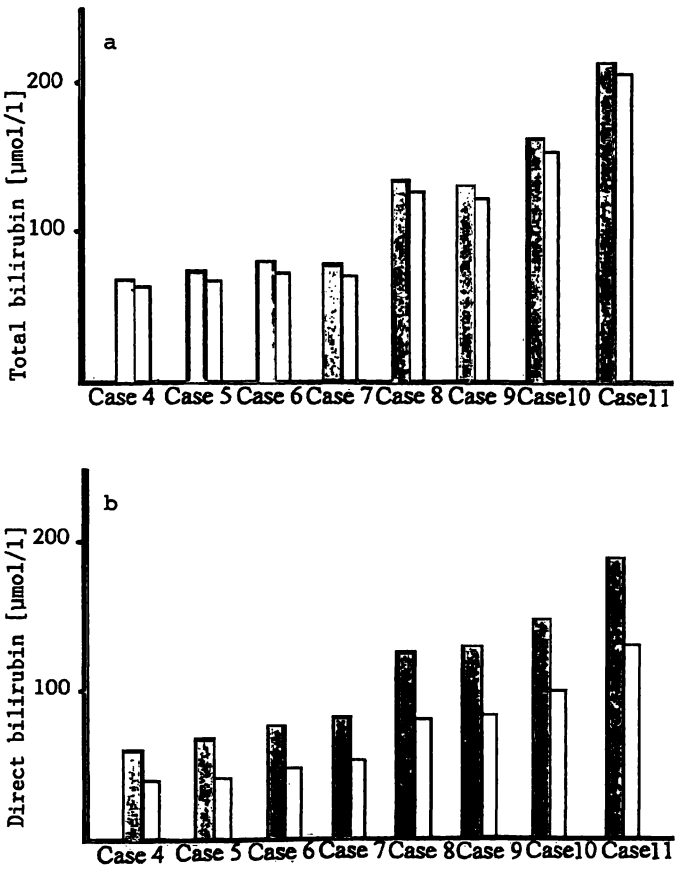

Fig. 2 Comparison of bilirubin values in type I hyperdirectbilirubinaemia.
(a) Total bilirubin values by the diazo method (■) and those by the enzymatic method (□).
(b) Direct bilirubin values by the diazo method (■) and those by the enzymatic method (□).
Mean difference (defined in the text) in direct bilirubin values was 53% whereas that in total bilirubin values was 7%.

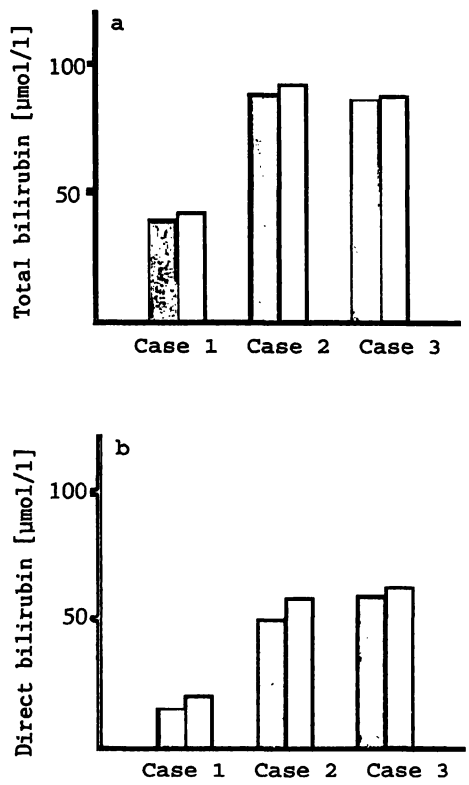

Fig. 3 Comparison of bilirubin values in type II hyperdirectbilirubinaemia.
(a) Total bilirubin values by the diazo method (■) and those by the enzymatic method (□).
(b) Direct bilirubin values by the diazo method (■) and those by the enzymatic method (□).
Mean difference (defined in the text) in direct bilirubin values was 14% whereas that in total bilirubin values was 3%.

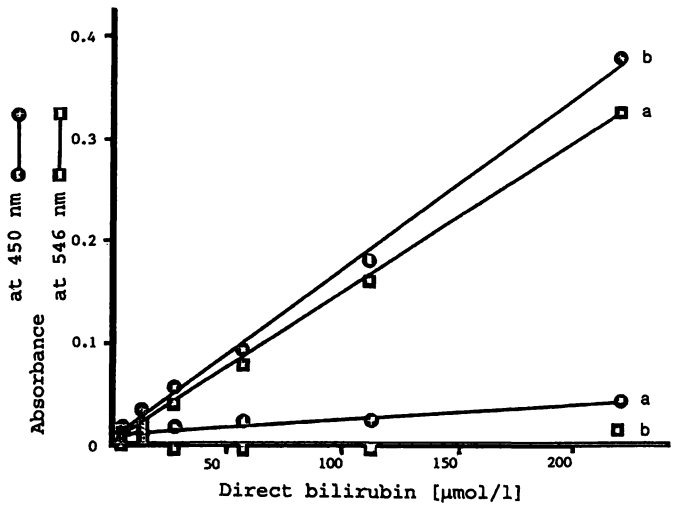

Fig. 4 Absorbance change before (b) and after (a) the reactions of the diazo (■—■) and the enzymatic (●—●) methods. Values by the diazo method were measured at 546 nm while those by the enzymatic method at 450 nm. The x-axis shows the direct bilirubin value of each sample measured by the diazo method.

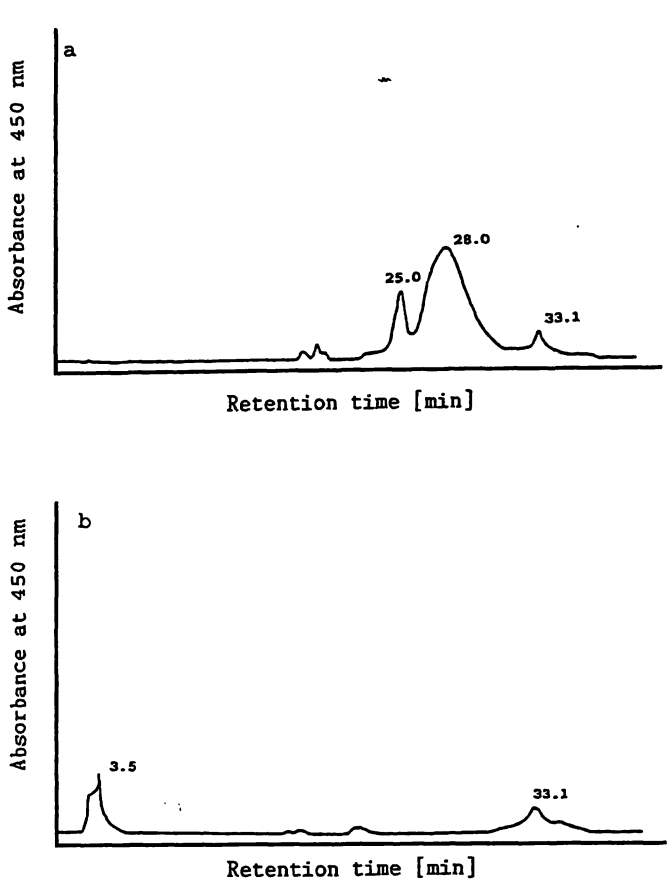

Fig. 5 Chromatogram of synthetic conjugated bilirubin before (a) and after (b) the addition of bilirubin oxidase at pH 3.7 (for direct bilirubin measurement).
The number at each peak indicates the retention time (min).

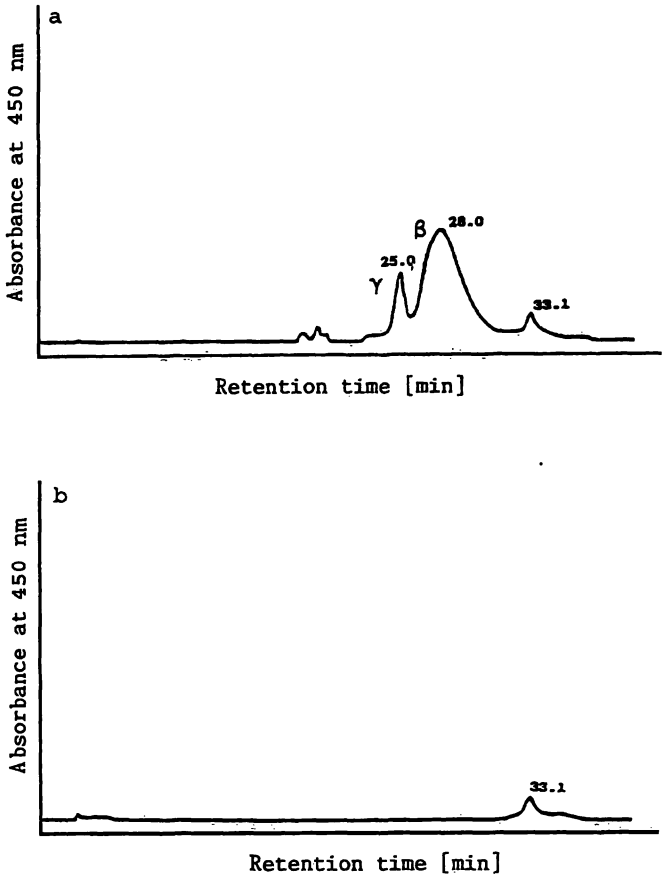

Fig. 6 Chromatogram of synthetic conjugated bilirubin before (a) and after (b) the addition of bilirubin oxidase at pH 7.2 (for total bilirubin measurement).
The number at each peak indicates the retention time (min).

min. At pH 7.2 (for total bilirubin measurement), this other material was not detected (fig. 6).

Discussion

It has been reported that measurement of δ bilirubin by the enzymatic method is not entirely accurate (9), and the same is true for the diazo method (13, 14). Thus, incomplete oxidation of δ bilirubin by bilirubin oxidase does not appear to be the main cause of the observed differences. In this analysis, type II hyperdirectbilirubinaemia (α and β were the main subfractions) showed little disparity (fig. 3), and unconjugated bilirubin (α subfraction) also showed little disparity between the two methods (data not shown). It is therefore evident that δ bilirubin is not responsible for the observed discrepancies.

In the present study HPLC was used to reconfirm the complete oxidation of conjugated bilirubin by bilirubin oxidase (fig. 5) as reported by *Lo* et al. (13) and to reveal that the disparity depends on relative proportions of β and γ subfractions. On the other hand the disparity could be accounted for by the fact that the baseline of the enzymatic reaction rose according to the concentration of synthetic conjugated bilirubin (ditaurobilirubin) (fig. 4). Also, HPLC analysis showed that the enzymatic reaction, when performed at pH 3.7, yielded material absorbing at 450 nm which had a retention time of 2.9 to 3.5 min (fig. 6b), but not when performed at pH 7.2 (complete oxidation conditions) (fig. 6b). It is suggested that this peak is due to incomplete oxidation products of ditaurobilirubin produced in the bilirubin oxidase reaction, since the absorbance increased in proportion to the ditaurobilirubin concentration and did not appear under the conditions of complete oxidation. Furthermore, the reactants include various substances absorbing at 450 nm on account of their broad peak width; but they do not include photobilirubin, one possible cause in the disparity, because this is attacked by bilirubin oxidase and its contribution to 450 nm absorbance is lost (15, 16).

Ditaurobilirubin is similar to conjugated bilirubin and serves as a suitable surrogate (17). Thus the main cause of the disparity is the incomplete oxidation of conjugated bilirubin by bilirubin oxidase. Bilirubin is completely oxidized if the pH in the reaction is raised. But the higher the pH, the more unconjugated bilirubin that is oxidized. Raising the pH is therefore problematic, so that complete

oxidation is difficult when measuring direct bilirubin. It is essential to take off this problem in the clinical interpretation of bilirubin values measured by the enzymatic method.

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